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HOST GENETICS OF SUSCEPTIBILITY

Toll-like receptor 1 variations influence susceptibility and immune response to *Mycobacterium tuberculosis*☆,☆☆

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SUMMARY

Background: Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* (MTB) infection, is still a global public health problem. TB susceptibility varies greatly in infected individuals, and mycobacterial recognition by the innate immune system likely affects disease course and outcome. This research describes a single nucleotide polymorphism in the Toll-like receptor (TLR) 1 gene that functionally alters the innate immune response to MTB and is associated with TB susceptibility in India.

Methods: 206 TB patients and 239 healthy controls from Hyderabad, India were analyzed for SNPs in the *TLR*1 and *TLR*2 genes, which were subsequently correlated to TB susceptibility. To test individual responses to MTB lysates, we stimulated PBMCs from genotyped healthy German individuals, as well as HEK cells transfected with TLR1/2 variants. TNF production and NF-kB activation were assessed respectively.

Results: Cohort analysis associated the *TLR*1-248N SNP (**RS4833095**) with TB protection. TLR1-248N expressing PBMCs from healthy controls exhibited an increased TNF response to MTB lysates. In addition to this, functional studies using HEK cell lines transfected with TLR1-248N and stimulated with MTB showed an increased NF-kB activation.

Conclusion: SNP TLR1-248N is associated with TB protection in an Indian population and exhibits an increased immune response to MTB lysate *in vitro*.

Abbreviations used: BCG, bacterium Calmette-Guérin; CI, Confidence interval; DNA, Deoxyribonucleic acid; ELISA, Enzyme-linked immunosorbent assay; HEK, Human embryonic kidney; LD, Linkage disequilibrium; LPS, Lipopolysaccharide; MTB, Mycobacterium tuberculosis; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; OR, Odds ratio; PBMC, Peripheral blood mononuclear cell; PCR, Polymerase chain reaction; RMSD, Root mean square deviation; SNP, Single nucleotide polymorphism; TB, Tuberculosis; TLR, Toll-like receptor; TNF, Tumor necrosis factor alpha.

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1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) is still a global public health problem that affects approximately 9 million people worldwide. The WHO estimates a third of the world's population to be infected with TB, of which only 5–10 % develop the disease. Variability in TB susceptibility was demonstrated by accident in 1926, when newborns in Lübeck, Germany were injected with live MTB instead of the vaccine *Bacillus Calmette-Guérin* (BCG). Some infants fell deadly ill while others showed no symptoms [1]. A clear genetic predisposition exists [2] and of the numerous candidate genes, members of the *Toll-like receptor* (TLR) family are of great interest.

TLRs are receptors of the innate immune system that recognize pathogen associated molecular patterns and initiate the immune response. Activation of TLRs leads to signal transduction and NF-κB activation, which ultimately results in the release of tumor necrosis factor alpha (TNF), an important cytokine for macrophage activation and granuloma formation during TB infection [3,4]. Among the 10 TLRs active in mammals, TLR2 recognizes microbial lipopeptides and can form a heterodimer with TLR1 or TLR6. The TLR2/1 heterodimer recognizes triacylated lipopeptides and the TLR2/6 complex is the receptor for diacylated lipopeptides [5,6]. The TLR2/1 complex interacts at an early time point with lipopeptides purified from *M. tuberculosis* and M. *leprae* [7,8], which is why we focused our studies on these receptors.

Genetic variations within the TLR signaling system have been described that contribute to disease susceptibility and severity, and these variations may have occurred due to selective pressure [9,10]. An altered ability to recognize MTB may contribute to an individual's TB susceptibility, and single nucleotide polymorphisms (SNPs) in the *TLR*1/*TLR*2-heterodimer likely influence this process [11,12]. In the case of TLR1/2, SNPs that affect the ligand-binding pocket have been shown to alter recognition of the causative agents for malaria [13], leprosy [14] and other diseases [11]. Regarding TB specifically, the *TLR*1-S602I (**RS5743618**) SNP has been associated with TB in several populations [15–17]. Studies on the Indian population are rare and findings are limited to the SNPs TLR1-S602I, TLR2-R753Q and a few SNPs in other TLRs [18].

In order to understand individual TB susceptibility, it is important to understand how TLR SNPs are associated with disease in specific populations, and the functional consequences of SNPs that alter TLR structure. Here we show an association between TLR1-248N and TB protection in southern India. Demonstrating functional relevance, we also show that the *TLR*1-248N SNP is associated with increased NF-κB activation and an increase in TNF production *in vitro*. Finally, computer modeling analysis suggests how this SNP may alter TLR1 receptor function.

2. Results

2.1. Cohort characteristics

206 TB patients and 239 controls, all HIV-negative, were enrolled in the study and general characteristics were recorded (Table 1): Mean age of patients and controls was 29.5 and 34.1 years respectively. The female to male ratio was 57.8 to 42.2 in patients and 50.2 to 49.8 in controls. As expected, the mean BMI was significantly lower amongst patients (18.8) as compared to controls (24.6). Significantly more people in our healthy control group had a BCG vaccination scar (83.3%) as compared to our patient group (52.4%; $P < 0.001$). Low income and drinking/smoking habits are risk factors for TB and these factors were also higher in our patient group.

Table 1
Demographic and clinical characteristics of tuberculosis patients and controls in Hyderabad, India.

Variable	TB patient (n = 206)	Control (n = 239)	P-value
Gender n (%)			
Male	87 (42.2)	119 (49.8)	0.11
Female	119 (57.8)	120 (50.2)	
Age years	29.5 (±9.8)	34.1 (±8.9)	< 0.001
BMI [kg/m^2]	18.8 (±4.5)	24.6 (±4.9)	< 0.001
BCG scar n (%)	108 (52.4)	199 (83.3)	< 0.001
Drinking n (%)	31 (15.0)	1 (0.4)	< 0.001
Smoking n (%)	46 (22.3)	18 (7.5)	< 0.001
Income [IRS/month]	7387 (±5170)	16381 (±22870)	<0.001
Married n (%)	69 (33.5)	150 (62.8)	< 0.001
Religion n (%)			
Hindu	88 (42.7)	75 (31.8)/	0.017
Muslim	117 (56.8)	160 (67.8)	
People in house	6.0 (±3.5)	5.5 (±2.7)	0.074

Data are presented as mean ± SD or %.

2.2. Novel TLR1 SNPs found in the Indian population

To investigate TLR SNP frequency, we PCR-amplified and sequenced a 1668 bp region of TLR1-Exon 4 and a 1410 bp region of TLR2-Exon 3 in all subjects (Supplementary Figure 1). Of the previously described polymorphisms in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.gov), 26 polymorphisms of TLR1 and ten of TLR2 were absent in our cohort. However, four novel TLR1 polymorphisms were identified TLR1-Q266Q (**SS981600212**), -E472K (**SS981600235**), -V502I (**SS981600253**) and -M516K (**SS981600272**), as shown in Figure 1A. Sequenced SNPs and their frequencies are shown in Supplementary Table 1, novel SNPs are indicated with an *.

2.3. TLR1-I602S and TLR1-N248S distribution differs in India, Germany and Africa

A clear genetic drift exists from TLR1-248S, which is predominantly present in Africa, to TLR1-248N, the most common genotype in Germany. In our Indian cohort, the two TLR1-248 variants occurred with similar frequency (Figure 1B). The SNP TLR1-602I, associated with TB risk in African-Americans [15], differs in frequency between Africa and Europe. In our Indian cohort, the homozygote 602S variant was present at a very low frequency, comparable to African cohorts (Figure 1C).

2.4. A synonymous SNP, TLR1 Q266Q is associated with TB risk

When comparing patient and control SNP frequencies, we found that one of the synonymous SNPs, TLR1 Q266Q, was associated with TB risk (odds ratio (OR) 0.05, 95% confidence interval (CI) 0.009-0.53, $P < 0.001$) (Supplementary Table 1). Linkage disequilibrium (LD) calculations, however, failed to reveal linkage with our sequenced TLR1 gene regions (Figure 2). The LD plot also indicates that TLR1-N248S and TLR1-I602S are not linked in our Indian cohort (5% correlation) (Figure 2), while they are strongly linked in our healthy German control cohort (82% correlation) (data not shown).

2.5. TLR1-248N is associated with protection against TB

Analysis of patients and controls demonstrated that the TLR1 SNP 248N (**RS4833095**) was associated with decreased TB susceptibility (Table 2; OR 0.69, 95% CI 0.53-0.9, $P = 0.036$; corrected for confounders: age, BMI, BCG vaccination status and smoking/drinking habits). Allele and genotype frequencies were in Hardy-Weinberg-Equilibrium. Genotype frequencies of SNP N248S were

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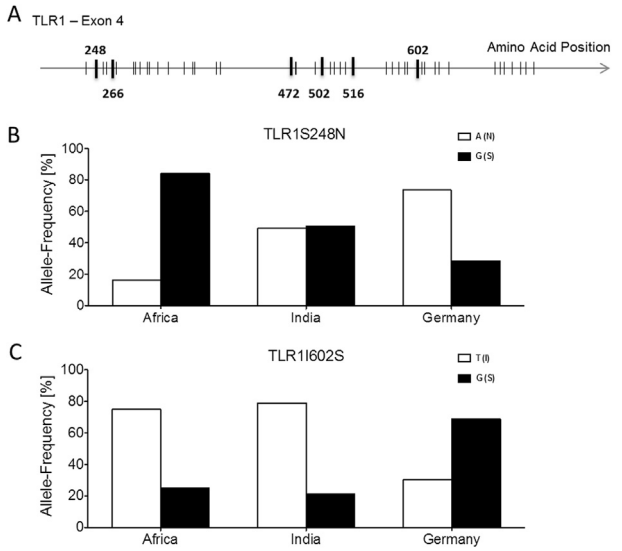

Figure 1. Comparison of TLR1 S248N and I602S SNP allele frequencies reveal genetic drift from Africa over India to Germany. A) The sequenced area of TLR1-Exon 4 on chromosome 4. Newly found polymorphisms are highlighted below and functionally analyzed SNPs above the arrow. B) The TLR1-248N variant is present at a low frequency in Africa, evenly distributed with the 248S variant in India and predominant in Germany. C) Regarding TLR1-I602S, the distribution changes between Africa/India and Germany. In this case, the 602S variant is even less frequently found in India as compared to Africa. Data from African populations were collected from the HapMap-Project, data from Indian population were collected during this study and German population data was previously collected in the Schumann lab.

further analyzed to determine if TB risk was associated with homozygous (742AA or 742 GG) or heterozygous (742AG) individuals. The frequency of the homozygote variant 742AA (248N) was significantly associated with a decreased TB risk (OR 0.57, 95% CI 0.37-0.88, P = 0.011) (Table 2). Allele 1805G (TLR1-602S), which has been associated with protection against TB in other populations [19,15], was present in only 8% of controls and 7% of patients and did not have an effect in our cohort.

2.6. The TLR1-248N allele contributes to increased PBMC TNF production

To investigate whether peripheral blood mononuclear cell (PBMC) reactivity is affected by the TLR1-248N allele, PBMCs were isolated from healthy German volunteers that were previously genotyped for the TLR1-248 and −602 SNPs. PBMCs were stimulated with mycobacterial ligands and TNF production was assessed in the supernatant by ELISA (Figure 3). Since the genotype combinations TLR1-248S-602S and TLR1-248N-602I are very rare in the German population, we limited our PBMC experiments to the analysis of individuals homozygous for TLR1-248S-602I (risk) and TLR-248N-602S (protective). As expected, the LPS negative control (a TLR4 ligand) resulted in no difference between groups (Figure 3A). PBMC stimulation with PAM_3Cys resulted in a slight increase of TNF production in TLR1-248N-602S individuals as compared with TLR-248S-602I individuals; however the difference was not significant (Figure 3A). Next, we stimulated with different HR37v MTB lysate concentrations to determine the dose response (Figure 3B). Importantly, we could show that TLR1-248N-602S PBMCs produced significantly more TNF than TLR1-248S-602I PBMCs ($P < 0.05$) when stimulated with H37Rv MTB whole cell lysate (Figure 3C). Though the PBMC experiments provided evidence that 248N is associated with increased TNF production, this single experiment was not conclusive due to the linkage with 602S. To provide more definite information, we then performed HEK cell stimulation experiments with all 4 combinations of TLR1-247 and −602 alleles.

2.7. The TLR1-248N variant results in increased HEK cell NF-κB expression

To better assess which TLR1 allele (248N or 602S) may have contributed to the increased TNF production in the PBMC

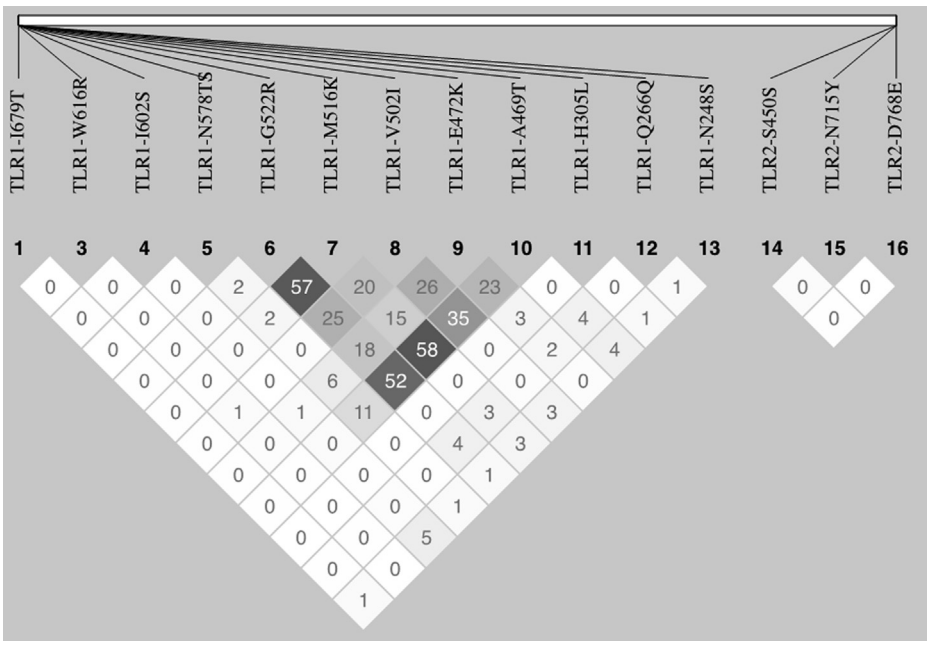

Figure 2. No linkage disequilibrium between TLR1-N248S, -S602I or -Q266Q. Linkage disequilibrium analysis of TLR1 SNPs (% correlation). A strong association is represented by a high percentage and darker squares. No association is observed between N248S and S602I (5% correlation). The novel TLR1 polymorphism Q266Q is not linked to any other SNP in the areas that were sequenced.

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Table 2
Genotype frequencies of the *TLR*1-N248S SNP in TB patients and healthy controls.

*TLR*1-N248S	TB patients n (%)	Controls n (%)	P-value	OR	95% CI
Allele					
G [248S]	229 (55.6)	222 (46.4)			
A [248N]	183 (44.4)	256 (53.6)	**0.0066**	**0.69**	**0.53–0.90**
*TLR*1-248N			**0.036***	**0.64**	**0.42–0.97**
Genotype					
AA vs AG + GG	42 (20.4)	74 (31.0)	**0.011**	**0.57**	**0.37–0.88**
AG vs AA + GG	99 (48.1)	108 (45.2)	0.69	1.47	0.97–2.24
GG vs AA + AG	65 (31.6)	57 (23.8)	0.54	1.12	0.77–1.63

Bold text indicates a significant P-value < 0.05.
* Adjusted for age, BCG, BMI, drinking, smoking.

experiment, we transfected human embryonic kidney (HEK 293) cells with all allelic combinations of TLR1-248 and −602, then measured NF-κB expression. Since HEK 293 cells lack functional TLRs, this was an ideal experimental system for our purpose. As expected, the LPS control did not differ from the un-stimulated control group and the positive PAM_3Cys control resulted in a significant up-regulation of NF-κB expression (Figure 4A). When stimulated with MTB HR37v lysate, cells transfected with the 248N allele (not the 602S allele) resulted in the highest NF-κB expression (Figure 4B).

2.8. Impact of the N248S variant on TLR1 structure

Next we analyzed the effect of TLR1-248 SNPs on protein structure *in silico*. Root mean square deviation (RMSD) calculations revealed that variant 248S had a deviation of 2.12 Å in C-alpha residues and 2.09 Å in the backbone atoms when compared to the template 248N. Comparison of CASTp results for 248N and 248S in TLR1 revealed that the amino acid 248N exists in one smaller pocket (Figure 5A) while the TLR1 variant 248S structure lacks this pocket (Figure 5B). Further analysis revealed the presence of 2 and 3 nests in 248N and 248S variants respectively. These concave nests are functionally relevant since they are known to house important motifs [20], however, none of these nests were found to be located in the vicinity of amino acid number 248. We found that the variant 248N differs significantly from 248S with respect to surface topography of the major clefts and cavities (Supplementary Table 2). One of these cavities is present in the 248N variant (Figure 5C, indicated in blue) and seems to be non-existent or not functional in 248S (Figure 5D). This cavity is able to host one part of the TLR1 agonist PAM_3Cys (Figure 5E). Due to the lack of this cavity in 248S, we hypothesize that PAM_3Cys recognition might be impaired in individuals with this genotype, possibly blocking TLR2/1 heterodimer formation (Figure 5F). The formation of this heterodimer allows PAM_3Cys to bind and to be recognized with the TLR1-248N variant with subsequent signal transduction (Figure 5G). With a lost cavity in TLR1-248S variant, PAM3Cys cannot bind and signal transduction cannot occur (Figure 5H). Additionally, if the cavity is moved in the TLR1-248S variant, the physical structure of the heterodimer might be drastically changed, also impairing TLR2/1 signal transduction (Figure 5I).

3. Discussion

We report a polymorphism in TLR1 (248N) that is associated with pulmonary TB protection in an Indian population (OR = 0.64, 95% CI = 0.42-0.97, P = 0.036). Although our study is limited by a small sample size, our results are in agreement with other larger studies of various populations. TLR1-248S was associated with tuberculosis susceptibility in an African American population [15] as well as leprosy in Bangledesh [21] and Brazilian populations [22]. Since *M. leprae* is a close relative of *M. tuberculosis*, these

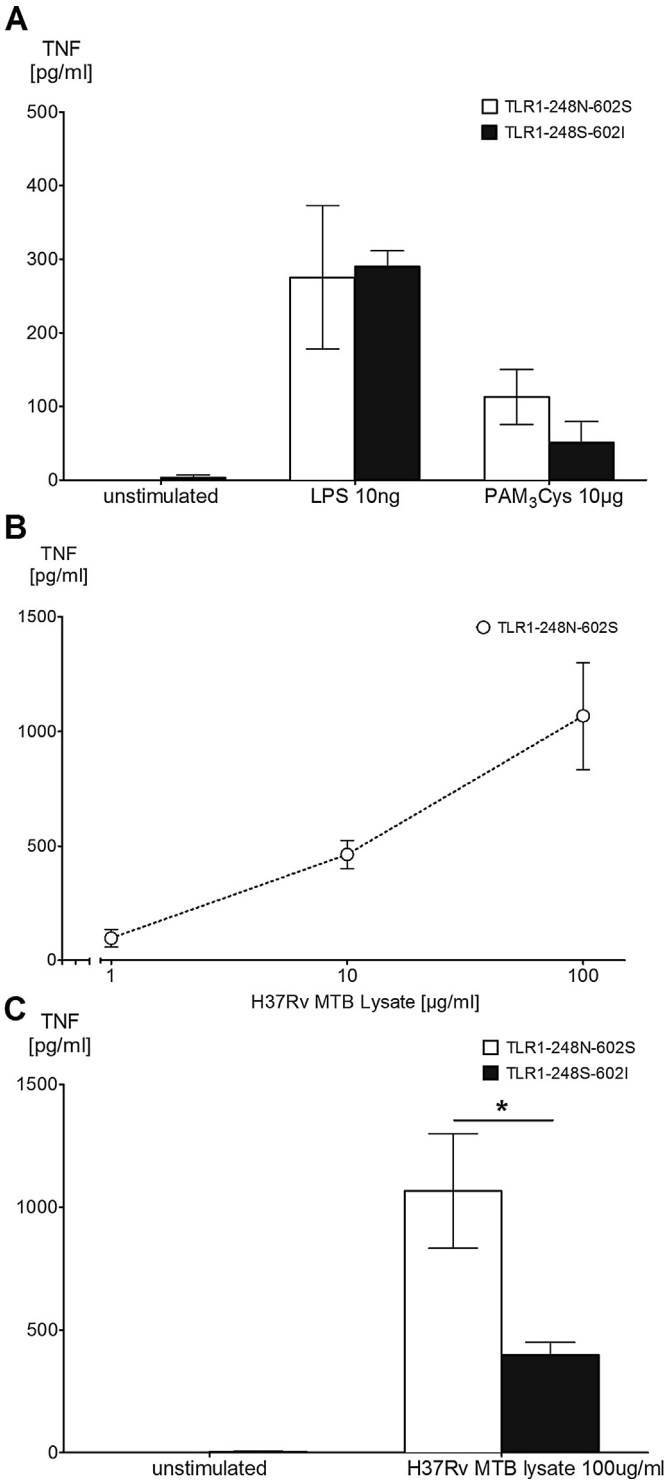

Figure 3. PBMCs from individuals with a TLR1-248N genotype exhibit a higher TNF response. PBMCs from were isolated from the whole blood of healthy German volunteers with homozygous genotypes TLR1-248N-602S or TLR1-248S-602I. A) Comparison of the TNF response between TLR1-248N-602S or TLR1-248S-602I homozygous genotypes; measured by ELISA of cell culture supernatant after stimulation with LPS and Pam_3Cys. B) Dose-response-curve of TNF production when TLR1-248N-602S PBMCs were treated with different concentrations of H37Rv MTB lysate. C) TNF response after stimulation of the 2 genotypes with 100 µg/ml of HR37v MTB whole cell lysate. *TLR*1-248N-602S exhibited significantly increased TNF production compared to TLR1-248S-602I. PBMCs from 4 age and sex matched individuals per genotype were individually plated with 3 technical replicates per individual. Results are representative of 2 experiments showing similar results Means ± SEM are shown. *, P < 0.05.

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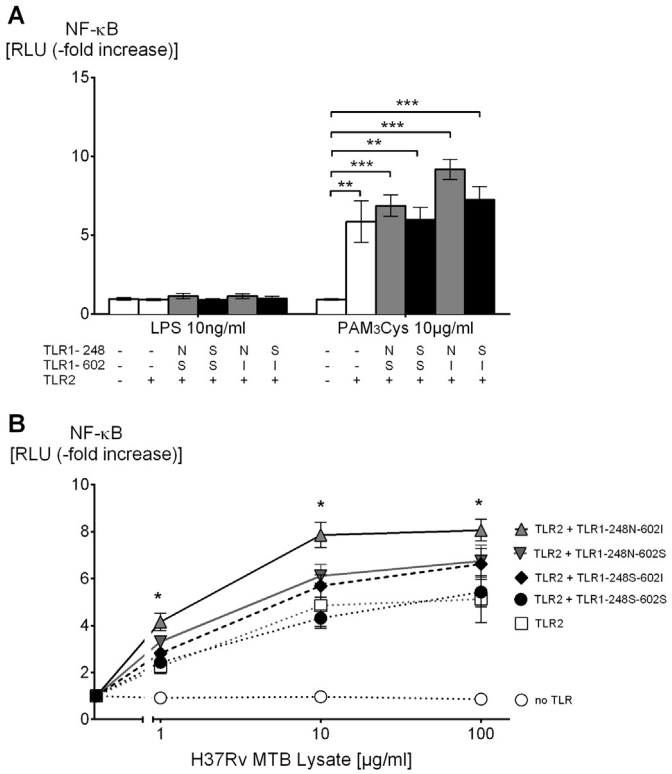

Figure 4. A significant increase in NF-kB activation is observed in HEK cells expressing TLR1-248N. Human embryonic kidney cells were transfected with *TLR2*, *TLR1*-S248N and *TLR1*-I602S variants. A) The NF-kB response of transfected cells was measured after LPS (negative control) and PAM_3Cys (positive control) stimulation. B) Dose-response-curve of all variations of the transfected cells when stimulated with HR37v MTB whole cell lysate. The *TLR1*-248N variant (not −602S) shows a significantly higher NF-kB response than all other allelic combinations. Values are normalized to their respective un-stimulated value and are visualized as -fold increase of the un-stimulated value. Results represent one out of 4 independently performed experiments with similar outcomes (n = 3 per group). Means ± SEM are shown. *, $P < 0.05$; **, $P < 0.01$, ***$P < 0.001$.

studies are all in support of our findings. Though several studies have found an association of TLR1-I602S with TB in African, Asian, European and Hispanic populations [15–17]; we hypothesize that no association was found in our study due to the low frequency of the TLR1-602S variant (0.5%) in our Indian population. This study also confirms the results of our Bangladesh leprosy study, where the TLR1-602S variant was almost absent [21].

The functional consequences of amino acid modification at TLR1-N248S when exposed to mycobacterial compounds are currently not known. 248S is associated with malaria risk [23], IgA nephropathy in Korean children [24] and mortality in gram-positive sepsis [25]; however it is also associated with a decreased prostate cancer risk [26]. To better understand the role of this SNP in TB pathogenesis, we utilized two cell culture systems to specifically examine the immune response to MTB lysate. In a first set of functional experiments, PBMCs from homozygous TLR1-248N–602S genotyped donors that were stimulated with MTB lysate showed a higher TNF response than TLR1-248S–602I donors.

To confirm that the results from our PBMC experiment were due to the 248N allele and not the commonly associated 602S allele, in a second experiment we transfected HEK cells with all TLR1-248 and −602 allelic combinations. The HEK cell experiments demonstrated that the TLR1-248N variant (associated with TB protection) resulted in significantly increased NF-kB expression after MTB stimulation. Transfections containing TLR1-602S variant did not result in significant increases in NF-kB expression. Interestingly, though Johnson et al. demonstrated that cells expressing the TLR1-602S variant were unable to express TLR1 on the cell membrane [14] we could still measure NF-κB activation in our transfected HEK cells. We argue that though 602S may result in reduction of TLR 1 cell surface expression, it does not necessarily result in TLR 1 inactivation. Evidence from the literature indicates that TLR1/2 can activate signaling cascades intracellularly from within endosomes [27]; this mechanism of TLR 1/2 activation is of interest for further investigations.

Although the most likely, and only unanimously proven ligand for activating the TLR2/1 complex is triacylated lipopeptide, our results using purified lipopeptide were less clear as compared to the results obtained by mycobacterial lysate. This may point to other, currently unknown TLR2/1 ligands present in the lysate with the potential to stimulate host cells in a TLR2/1-dependent fashion. Other groups, quite interestingly both originating from India, have recently proposed mycobacterial proteins to be stimulators of the TLR2/1 complex; antigens termed Rv1196 that are members of the cell surface PPE family of proteins [28], or the proteins termed Rv0978c and Rv0754 (members of the cell surface PE-PGRS family of proteins) [29]. Usually, however, these proteins were recombinantly expressed in bacterial systems, which carry the risk of bacterial lipopeptide contamination as has been commented on by others [30].

In addition to demonstrating that the protective TLR1-248N SNP was associated with higher TNF production in PBMCs and increased NF-κB expression in HEK cells, we used *in silico* modeling to determine how amino acid changes at position 248 might affect protein structure. From our findings, it is evident that 248N and 248S greatly alter TLR1 structure. Bioinformatics analyses revealed a potential functional pocket at 248N that is lost in the 248S variant. An extra functional binding pocket could allow stronger ligand-receptor binding; indeed, our functional studies demonstrate that cells expressing the 248N SNP had a stronger activation than 248S. A similar trend was noted in the predicted cleft size at position 248. Interestingly, the cleft created by the 248N SNP is substantially larger than the 248S cleft. Since, clefts are known to be biologically important, with larger clefts housing more binding regions and active sites [31], it follows that the large cleft at the 248N site could also account for stronger receptor-ligand binding and thus stronger activation of cells expressing this TLR1 variant. Models investigating triacylated lipopeptide PAM_3Cys binding also demonstrated that the established cleft and binding site in TLR1-248N moves to a completely different location in the 248S variant. The shifted cleft location indicates structural changes that could influence either TLR1/2 heterodimer formation or signal transduction.

To summarize, we were able to show that TLR1-248N is associated with protection against TB in an Indian population. Functional analysis with both PBMC and HEK cell culture stimulated with MTB lysate revealed that cells expressing the TLR1-248N allele produced more TNF and had higher NF-kB expression. *In silico* modeling showed structural differences between the 248N and the 248S variants, indicating that the mutated 248S variant has impeded possibilities to bind important ligands such as lipopeptides. Taken together, these findings suggest an important role for the TLR1-N248S polymorphism in TB infection and have provided insight into how this SNP participates in TLR1 structure and functionality. Genotyping of individuals could potentially help identify high risk groups, which may lead to improved and individualized strategies of prevention and therapy of TB.

4. Study population, materials & methods

Patients and Controls For this case–control study, we enrolled 206 pulmonary TB patients and 239 healthy controls in Hyderabad, India. All patients and controls were Indians living in or around the

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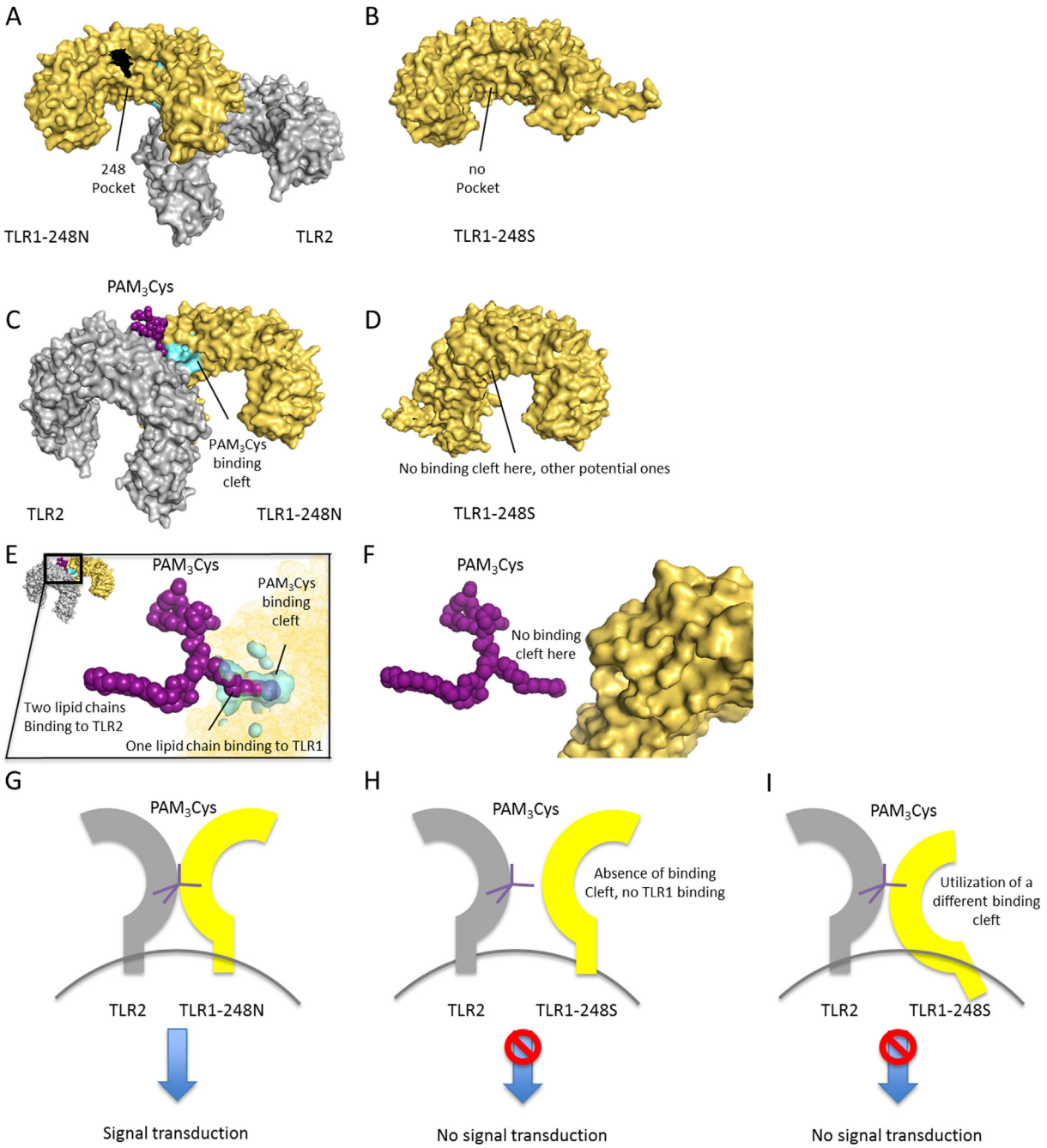

Figure 5. A possible functional binding pocket in the TLR1-248N variant, associated with protection against TB, is lost in the 248S variant. A) The model created from the crystal structure of the TLR1/2 heterodimer shows a potential binding pocket including amino acid position 248 in the TLR1 portion of the heterodimer (TLR1 – yellow, TLR2 – gray, PAM_3Cys – purple). B) We calculated a model incorporating the 248S variant. Here, the possible binding pocket is absent. C) The other side of Model A shows the cavity indicated in blue, where the ligand PAM_3Cys (purple) can slide into the pocket in TLR1. D) Again, in the mutated (248S) version, this cavity seems to be closed or moved. E) Close-up of PAM_3Cys (purple) docked in the cavity (blue) of TLR1-248N. F) Close-up of TLR1-248S and PAM_3Cys (purple), which cannot move into the closed cavity. G) Illustration of the TLR1/2 heterodimer on the cell surface binding PAM_3Cys. H) Illustration of how a closed cavity in TLR1-248S could interfere with PAM_3Cys binding and subsequent signal transduction. I) Illustration of how a different PAM_3Cys binding cleft in TLR1-248S, could alter TLR1/2 heterodimer formation and subsequent signal transduction.

city of Hyderabad, India. Patients, who attended the Free Chest TB Clinic PPM DOTS at Mahavir Hospital and Research Centre, Hyderabad, were confirmed with sputum microscopy for AFB and chest X-ray or histopathology as per the guidelines of the Revised National Tuberculosis Control Program (RNTCP). Additionally, a tuberculin skin test (TST) was carried out by administering five tuberculin units of purified protein derivative of whole MTB (PPD) sub-cutaneous on the left arm. Since individuals are residents in a high-risk area, an induration of >10 mm in between 48 and 72 h was considered positive. Household contacts and healthy controls of the same region with no history of tuberculosis were also enrolled in the study. All subjects were HIV-negative. Procedures used in the study conformed to the principles outlined in the Declaration of [32]. All samples were collected with the written and informed consent of the participants. The study protocols were approved by the institutional ethics committee of Mahavir Hospital

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Hyderabad and Charité Medical University Berlin. The German control cohort used for this study was previously described by Oh et al. [33].

DNA Prep/Sequencing/PCR Specific regions of TLR1 - Exon 4 (1668 bp) and TLR2 - Exon 3 (1410 bp) were chosen for sequencing based on 1) the impact of known SNPs on TLR function and 2) the involvement of particular TLR SNPs in infectious diseases. Sequencing was performed, rather than single SNP analysis, due to the possibility of identifying new SNPs in our regions of interest. Genomic DNA was prepared from the whole blood of TB patients and healthy volunteers using a DNA Blood mini kit (Qiagen GmbH, Hilden, Germany) or from cheek cells using a DNA kit (Qiagen). For more detail regarding PCR amplified regions in TLR1 and TLR2, please see Supplementary Figure 1.

The primers for PCR amplification and sequencing for *TLR*1 were: TLR1A 5′-TGGTCTTAGGAGAGACTTATGGG-3′ and 5′-GCTTACA-GAATTCTGGCTAATATCC-3′, TLR1B 5′-GCACCTTACTGAGTTGGAGA-CAC-3′ and 5′-GAGATACCAGG GCAGATCCAAG-3′, TLR1C 5′-CAACATAACTCTGCTGATCGTCAC-3′ and 5′-AGGAATGGA GTACTGCG-GAATG-3′ and for *TLR*2: TLR2A 5′-TTCTGGTTCCTTGTTTACTTTCAC-3′ and 5′-GCTGTCCTGTGACATTCCG-3′ and TLR2B 5′-TCTTGATT-GATTGGCCAGC-3′ and 5′-AAATGACGGTACATCCACGTAG-3′ (Eurofins Genomics, India Pvt. Ltd). PCR amplification ran for 34 cycles with a master mix containing 7.5 μl Go-Taq Hot Start Green Master Mix (Promega GmbH, Mannheim, Germany), 0.5 μl H_2O, 0.5 μl forward primer (10 pmol/μl), 0.5 μl reverse primer (10 pmol/μl) and 6 μl of genomic DNA (10 ng/μl). Samples were checked on a 1% agarose gel, and then purified using a Qiaquick PCR purification kit (Qiagen). For SNP detection, the DNA samples were sequenced (Ocimum Biosolutions Ltd., Hyderabad, India) using Sanger sequencing (Sequencer: Applied Biosystems 3730xl DNA Analyzer, Life Technologies Corporation, Carlsbad, California, USA) with the same primers as for PCR amplification. The sequencing reads were analyzed using Lasergene 11 (DNAStar Inc, Madison, WI, USA), Consed 24.0 [34], Phred build 071220 and Phrap 1.09 [35]. Haplotype analysis was done with Haploview [36]. Accession numbers for all DNA sequences and polymorphisms in this work are **NM_003263.3**/**NP_003254.2**.

*Plasmids TLR*1 PEF6 V5 plasmids were kindly provided by Dr. Tom Hawn (University of Washington, Seattle, WA, USA) and received on filter paper which was soaked for 30 min in 50 μl of Qiagen Elution Buffer. Resulting suspensions were transformed in NEB 10-beta competent *E. coli* (New England Biolabs, Ipswich, MA, USA) following the manufacturer's protocol. Resulting colonies were picked and incubated overnight at 37 °C in 5 ml LB-medium + 5 μl ampicillin. Plasmids were extracted using a Qiagen Miniprep Kit. DNA concentration was measured using a ScanDrop 250 (Analytic Jena AG, Jena, Germany) and a restriction digestion was performed. Plasmid-containing bacteria were incubated overnight at 37 °C shaking at 200 rpm in LB-medium + 0.1% ampicillin. Maxipreps were performed to purify plasmid DNA (Machery-Nagel GmbH & Co. KG, Düren, Germany).

Functional Analysis: Stimulation of PBMCs from healthy volunteers Blood was taken from volunteers from a previously described German control cohort [33] who had been genotyped as homozygous for the *TLR* SNPs of interest. Blood was collected in EDTA tubes (Becton Dickinson GmbH, Heidelberg, Germany) and PBMCs were separated with Ficoll (GE Healthcare Europe GmbH, Freiburg, Germany), centrifuged (1200 × *g*, 20 min, 20 °C) and washed three times with RPMI medium. PBMCs from 4 age and sex matched individuals per genotype were individually plated with 3 technical replicates per individual. Results are representative of 2 experiments showing similar results. In a 96 well flat bottom plate 2×10^5 cells were stimulated with Pam_3Cys (EMC microcollections GmbH), LPS or H37Rv Whole Cell Lysate *M. tuberculosis* (BEI Resources) for 4 h and TNF was measured in the supernatant by ELISA (BD Biosciences, Heidelberg, Germany).

ELISA Nunc Maxi Sorp ELISA plates were coated with 5 g/ml rabbit anti-hTNF antibody, diluted in 100 mM NaHCO3, pH 8.3 at 4 °C overnight. After blocking with PBS plus 0.05% Tween 20 (Sigma) and 10% fetal calf serum (FCS, Invitrogen) for 2 h at room temperature, samples and recombinant TNF (R&D, Wiesbaden, Germany) were added and incubated at 4 °C overnight. After washing, biotinylated anti-hTNF antibody (BD Pharmingen, Hamburg, Germany) at 5 g/ml was added and incubated at room temperature for 1 h, followed by incubation with streptavidin peroxidase (1 g/ml, Sigma) for 30 min. Detection of bound TNF was carried out with ortho-phenylen-diphosphate (OPD, Sigma) followed by measurement at 490 nm in an ELISA reader (Tecan, Crailsheim, Germany).

HEK NF-kB reporter gene assay HEK-Blue Null1 Cells (InvivoGen, Toulouse, France) were cultured in Dulbecco-MEM Medium (Life Technologies GmbH, Darmstadt, Germany) and transfected the next day with beta-gal, ELAM, *TLR*1 (248N-602I, 248N-602S, 248S-602I and 248S-602S) and *TLR*2 (wild type) plasmids. After 24 h, cells were washed and 2.8×10^5 cells were stimulated in a 96-well flat bottom cell culture plate for 24 h with Pam_3Cys (EMC microcollections GmbH, Tübingen, Germany), LPS or H37Rv Whole Cell Lysate *M. tuberculosis* (BEI resources). NF-kB stimulation was determined using HEK-Blue detection (Invivogen) as per manufacturer's protocol.

Statistics Statistical analysis was performed using SPSS Statistics Version 21 (IBM, New York, NY, USA), Prism (GraphPad Software Inc, La Jolla, CA, USA) Version 5 and R (R Foundation for Statistical Computing, Vienna, Austria). Allele and genotype frequencies were estimated by direct counting and the χ^2 test was used. For the association of SNPs with TB susceptibility either a Pearson Chi-square or Fisher exact two-tailed test was used (the specific test used is indicated in Supplementary Table 1). Adjusted P-values were calculated with binary logistic regression using SPSS. For the PBMC analysis of normally distributed data, a Student's T-test was used. For HEK cell experiments the Mann–Whitney U test was used.

Modeling The three dimensional protein structures of the TLR1-248 variants were generated by homology modeling using MODELLER 9.12 (Mac version) [37] on the basis of its alignment and homology with the crystal structure of TLR1 (PDB ID: 2Z7X). To remove erroneous bad sectors between the atoms the crude model was subjected to refinement through energy minimizations using Swiss PDB Viewer 4.1 package [38]. Steepest descent and conjugate gradient methods were applied with GROMOS96 43B1 force field parameters. The final model was evaluated with SAVES (http://nihserver.mbi.ucla. edu/SAVES/) using PROCHECK, WHATCHECK, ERRAT and PROVE. ProSA [39] was used to determine the stereo chemical quality and reliability of the modeled structure. RMSD calculations were performed with Swiss PDB viewer to indicate the difference between variants. ProFunc [40] was used to predict functional nests, clefts and cavities. CASTp [41] was used to predict pockets in the structure. Visualizations were done with PyMOL v1.6.0.0 Enhanced for Mac OS X (www.pymol.org).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2015.02.045

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